

***Remarks***

The amendment presented above is sought to place the application in condition for allowance and/or into better form for appeal. *See* 37 CFR § 1.116(a). A request for continued examination (RCE) is being filed concurrently herewith; therefore, the finality of the Office Action dated September 19, 2001 should be withdrawn. *See* 37 CFR § 1.114(d). The amendment presented above introduces no new matter and presents no new issues requiring further consideration or search. It is therefore respectfully requested that the amendment after final action be entered. *See* 37 CFR § 1.116(a).

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 45-49 and 52 are pending in the application, with claim 45 being the sole independent claim.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

***I. Support for Amendments***

Support for the amendments to claim 45 can be found, *inter alia*, in the specification at page 39, lines 13-27, and in original claim 45.

***II. Summary of the Office Action***

In the final Office Action dated September 19, 2001, the Examiner has made two rejections to the claims. Applicants respectfully offer the following remarks to overcome each of these rejections.

**III. Claim Rejections Under 35 USC § 112, Second Paragraph**

The Examiner has rejected claims 45-49 and 52 under 35 USC § 112, second paragraph, as being indefinite for allegedly failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. *See* Paper No. 6, page 2, item 4. Applicants respectfully traverse this rejection.

The Examiner has based the rejection under 35 USC § 112, second paragraph, on the recitation in claim 45 of the phrase "wherein said composition does not comprise a nucleic acid molecule." According to the Examiner, "it is unclear as to whether applicants intent is to require a composition in which all types of nucleic acids are absent, whether applicants intent is for the claims to encompass any composition in which any particular 'nucleic acid molecule' is absent, etc." Paper No. 6, page 3.

Applicants have deleted from claim 45 the phrase "wherein said composition does not comprise a nucleic acid molecule." Thus, the rejection under 35 USC § 112, second paragraph is moot and should accordingly be withdrawn.

**IV. Claim Rejection Under 35 USC § 102**

The Examiner has also rejected claims 45-49 and 52 under 35 USC § 102(e) as being anticipated by Chenchik *et al.*, U.S. Patent No. 5,565,340 ("Chenchik"). *See* Paper No. 6, page 3, item 6. Applicants respectfully traverse this rejection.

The Examiner has set forth two bases to support the rejection. First, the Examiner asserts that, even though (some of) the compositions disclosed in Chenchik include nucleic acid molecules, such compositions may nonetheless be regarded as not comprising a nucleic acid molecule because "most nucleic acid molecules known in the art are in fact absent from

those compositions." *See* Paper No. 6, page 4. These comments relate specifically to the phrase "wherein said composition does not comprise a nucleic acid molecule" in claim 45. As discussed above, however, Applicants have deleted this phrase from claim 45. Thus, the Examiner's first basis for the rejection under 35 USC § 102(e) is moot.

The Examiner's second basis of rejection under 35 USC § 102(e) is that Chenchik "inherently disclose[s] compositions comprising a restriction endonuclease and a polymerase inhibitor in which nucleic acids are absent." *See* Paper No. 6, page 4. More particularly, the Examiner points to Example 2 in Chenchik (column 20, line 38, through column 21, line 32), wherein products of a PCR reaction were examined on a "1.2% agarose/ethidium bromide (EtBr) gel." It is the Examiner's belief that, by analyzing the PCR products on an agarose gel (by electrophoresis), the nucleic acid molecules were removed from a composition comprising one or more restriction endonucleases and one or more polymerase inhibitors, thereby producing a composition meeting the limitations of Applicants' claims. *See* Paper No. 6, page 4. According to the Examiner:

It is well known to those of ordinary skill in the art that such a display of digested PCR products results in migration of the nucleic acids present in a liquid amplification product sample into semisolid, gel material. As a result of this migration of nucleic acids from liquid into gel, the remaining liquid composition lacks the presence of nucleic acids. Accordingly, Chenchik *et al* inherently disclose a liquid composition lacking the presence of nucleic acids, which liquid composition also meets the limitations of the present claims.

*Id.* Applicants respectfully disagree with this assessment.

A careful reading of the Chenchik disclosure reveals that the composition that was applied to the agarose gel did *not* include one or more restriction endonucleases. As stated

in Chenchik, after human genomic DNA was digested with restriction enzymes, "[t]he DNA was extracted once with phenol/chloroform/isoamyl alcohol (25/24/1) and precipitated by addition of 1/10th volume 3M NaOAc, 20 µg of glycogen and two volumes of 95% ethanol." See Chenchik, column 20, lines 52-55. As understood by those skilled in the art, the extraction of a DNA solution with phenol/chloroform/isoamyl alcohol functions to remove proteins, such as restriction endonucleases, from the solution. See Sambrook *et al.*, "Molecular Cloning, A Laboratory Manual," 2<sup>nd</sup> Ed., Cold Spring Harbor Laboratory Press (1989), pages E.3-E.4 (copy enclosed herewith as Exhibit A). Thus, the nucleic acid preparation that was subsequently used in the ligation and PCR reactions disclosed in Chenchik was devoid of restriction endonucleases.

Accordingly, even assuming *arguendo* that the Examiner's interpretation of Chenchik's Example 2 was accurate (*i.e.*, that gel separation effectively removed the nucleic acid molecules from the other components of the solution), the composition that remained after separation of the PCR products could not have contained one or more restriction endonucleases because there were no proteins, *i.e.*, *no restriction endonucleases*, in the composition that was applied to the agarose gel in the first place. Thus, the resulting composition could not anticipate Applicants' claims.

Notwithstanding the above discussion, Applicants note that claim 48 in particular is not anticipated by the Chenchik disclosure in any event. Claim 48 specifies that the polymerase inhibitor included within the composition is an antibody selected from the group consisting of an anti-*Taq* antibody, an anti-*Tne* antibody, an anti-*Tma* antibody, an anti-*Pfu* antibody and fragments thereof. The antibody used in Chenchik's Example 2, which the Examiner relies upon to support the § 102(e) rejection, was an anti-*Tth* antibody. Since

Chenchik does not disclose a composition comprising one or more endonucleases *and one or more of the antibodies, or fragments thereof, that are specified in claim 48*, Chenchik cannot and does not anticipate claim 48.

In view of the forgoing discussion, Chenchik clearly does not disclose all of the elements of Applicants' claims. Accordingly, Applicants respectfully request that the rejection of claims 45-49 and 52 under § 102(e) be reconsidered and withdrawn.

### ***Conclusion***

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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45. (Twice amended) A composition comprising one or more restriction endonucleases and one or more polymerase inhibitors[, wherein said composition does not comprise a nucleic acid molecule].

## **PURIFICATION OF NUCLEIC ACIDS**

Perhaps the most basic of all procedures in molecular cloning is the purification of nucleic acids. The key step, the removal of proteins, can often be carried out simply by extracting aqueous solutions of nucleic acids with phenol:chloroform and chloroform. Such extractions are used whenever it is necessary to inactivate and remove enzymes that are used in one step of a cloning operation before proceeding to the next. However, additional measures are required when nucleic acids are purified from complex mixtures of molecules such as cell lysates. In these cases, it is usual to remove most of the protein by digestion with proteolytic enzymes such as pronase or proteinase K (see Appendix B, Table B.9), which are active against a broad spectrum of native proteins, before extracting with organic solvents.

### ***Extraction with Phenol:Chloroform***

The standard way to remove proteins from nucleic acid solutions is to extract first with phenol:chloroform and then with chloroform. This procedure takes advantage of the fact that deproteinization is more efficient when two different organic solvents are used instead of one. Furthermore, although phenol denatures proteins efficiently, it does not completely inhibit RNAase activity, and it is a solvent for RNA molecules that contain long tracts of poly(A) (Brawerman et al. 1972). Both of these problems can be circumvented by using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1). The subsequent extraction with chloroform removes any lingering traces of phenol from the nucleic acid preparation. Extraction with ether, which was widely used for this purpose for many years, is no longer required for routine purification of DNA.

1. Add an equal volume of phenol:chloroform to the nucleic acid sample in a polypropylene tube with a plastic cap.

The nucleic acid will tend to partition into the organic phase if the phenol has not been adequately equilibrated to a pH of 7.8–8.0.

2. Mix the contents of the tube until an emulsion forms.
3. Centrifuge the mixture at 12,000g for 15 seconds in a microfuge (or at 1600g for 3 minutes in another rotor) at room temperature. If the organic and aqueous phases are not well-separated, centrifuge again for a longer time or at a higher speed.

Normally, the aqueous phase forms the upper phase. However, if the aqueous phase is dense because of salt ( $>0.5$  M) or sucrose ( $>10\%$ ), it will form the lower phase. The organic phase is easily identifiable because of the yellow color contributed by the hydroxyquinoline that is added to phenol during equilibration (see Appendix B).

4. Use a pipette to transfer the aqueous phase to a fresh tube. For small volumes ( $<200$   $\mu$ l), use an automatic pipettor fitted with a disposable tip. Discard the interface and organic phase.

To achieve the best recovery, the organic phase and interface may be "back-extracted" as follows: After the first aqueous phase has been transferred as described above, add



an equal volume of TE (pH 7.8) to the organic phase and interface. Mix well. Separate the phases by centrifugation as in step 3. Combine this second aqueous phase with the first, and proceed to step 5.

5. Repeat steps 1 through 4 until no protein is visible at the interface of the organic and aqueous phases.
6. Add an equal volume of chloroform and repeat steps 2 through 4.
7. Recover the nucleic acid by precipitation with ethanol as described on page E.10.

Occasionally, ether is used to remove traces of chloroform from preparations of high-molecular-weight DNA (see Note below).

#### Note

The organic and aqueous phases may be mixed by vortexing when isolating small DNA molecules (<10 kb) or by gentle shaking when isolating DNA molecules of moderate size (10–30 kb). When isolating large DNA molecules (> 30 kb), the following precautions must be taken to avoid shearing (see also Chapter 9).

- The organic and aqueous phases should be mixed by rotating the tube slowly (20 rpm) on a wheel.
- Large-bore pipettes should be used to transfer the DNA from one tube to another.
- The DNA should not be precipitated with ethanol (step 7). Instead, traces of chloroform should be removed either by extensive dialysis of the DNA solution against large volumes of ice-cold TEN or by extraction with water-saturated ether as described below.

**Caution:** Ether is highly volatile and extremely flammable and should be used and stored only in an explosion-proof chemical hood.

- a. In a large glass bottle, add water to the ether and mix well. Continue to add water until additional water does not enter the ether phase and forms a layer on the bottom of the bottle.
- b. Combine the DNA sample with an equal volume of the water-saturated ether prepared in step a and mix. Let the organic and aqueous phases separate by allowing the solution to stand for 2–5 minutes.
- c. Remove and discard the upper layer (ether is less dense than water).
- d. Repeat steps b and c.
- e. Remove traces of ether by heating the DNA solution to 68°C for 5–10 minutes with gentle mixing or by blowing a stream of nitrogen gas over the surface of the solution for 10–30 minutes.

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# ***Molecular Cloning***

A LABORATORY MANUAL  

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SECOND EDITION

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# **Molecular Cloning**

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